NOTES

Phaseolotoxin-Insensitive Ornithine Carbamoyltransferase of Pseudomonas syringae pv. phaseolicola: Basis for Immunity to Phaseolotoxin

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Cell-free extracts from phaseolotoxin-producing strains of Pseudomonas syringae pv. phaseolicola grown at 18°C, the optimum temperature for phaseolotoxin production, contain an ornithine carbamoyltransferase activity that is insensitive to phaseolotoxin. Extracts from the same strains grown at 30°C, a temperature at which little or no detectable phaseolotoxin is produced, and from phaseolotoxin-nonproducing strains contain a phaseolotoxin-sensitive ornithine carbamoyltransferase activity. The phaseolotoxin-insensitive ornithine carbamoyltransferase activity is also less sensitive to N8-(phosphonacetyl)-L-ornithine than the phaseolotoxin-sensitive ornithine carbamoyltransferase activity of the corresponding strain.

Phaseolotoxin [(N8-phosphosulfamyl)-ornithylalanylhomoarginine] (5), a tripeptide with phytotoxic (5, 8) and antibacterial (10, 14) properties produced by toxigenic strains of Pseudomonas syringae pv. phaseolicola (16), is a potent inhibitor of ornithine carbamoyltransferase (OCTase, EC 2.1.3.3) from plant, mammalian, and bacterial sources (7; A. R. Ferguson and J. S. Johnson, Physiol. Plant Pathol., in press; our unpublished data). In bacteria such as Escherichia coli and Salmonella typhimurium (10, 14) and in plant tissue culture (this laboratory; Z. R. Sung, unpublished data), phaseolotoxin causes a phenotypic requirement for arginine. Supplementation of this amino acid or its biosynthetic precursor, citrulline, to the culture medium reverses growth inhibition of E. coli and S. typhimurium (14). Citrulline supplementation also reverses growth inhibition of carrot tissue cultures (this laboratory; Sung, unpublished data). In contrast to E. coli and S. typhimurium, phaseolotoxin-producing strains of P. syringae pv. phaseolicola are insensitive to phaseolotoxin and have no natural growth requirement for arginine or citrulline. The biochemical basis of this insensitivity has not been previously reported. In this paper we present preliminary data on the detection of an unusual OCTase activity in extracts of phaseolotoxin-producing strains of P. syringae pv. phaseolicola that is resistant to phaseolotoxin and may, therefore, play a key role in the mechanism by which these strains are insensitive to their own toxin.

The strains used in this study have been described previously (14) and included E. coli N100 and eight P. syringae pv. phaseolicola strains, of which five produced phaseolotoxin (HB16, HB31, HB33, HB36, and NPPH3007) and three did not (HB13, HB20, and HB2). As reported elsewhere (6, 14), phaseolotoxin-producing (toxigenic) strains of this bacterium, but not those which do not produce the compound, cause chlorosis in inoculated leaves of Phaseolus vulgaris at low, but not at high, temperatures (optimal at 18°C). In the former group of strains, phaseolotoxin is produced optimally during growth at 18°C, whereas at 30°C, little or no phaseolotoxin is made (6, 14). Strains which do not cause chlorosis produce no or trace amounts of phaseolotoxin. Purified phaseolotoxin and filtrates from 18°C cultures of toxigenic strains inhibit the growth of E. coli or S. typhimurium strains with a functional oligopeptide permease (Opp+)(10, 15). Inhibition of E. coli or S. typhimurium by phaseolotoxin is not observed in strains lacking a functional oligopeptide permease (Opp−)(10, 15).

Phaseolotoxin was purified from P. syringae pv. phaseolicola PDDCC4419 (6) grown in minimal salts-glucose medium at 18°C (14). Phaseolotoxin was extracted from the supernatant fluid (12,000 × g, 15 min) by the method of Mitchell (5) and modified by us (Staskawicz and
Panopoulos, manuscript in preparation) in that the toxin was bioassayed by the *E. coli* bioassay (14) during purification. The final preparation was active in the leaf chlorosis assay, inhibited OPP' strains of *E. coli* and *S. typhimurium* (10, 15), and gave a positive test for phosphate (1). Its chromatographic and electrophoretic behavior on cellulose MN300 thin-layer plates was identical to that of phaseolotoxin (5). It was susceptible to L-amino peptidase (EC 3.4.11.1) treatment, yielding three ninyhydrin-positive products which cochromatographed with N\(^2\)-(phosphonacetyl)-L-ornithine, alanine, and homoarginine (5, 15). L-Amino peptidase treatment destroyed its antibacterial activity. The preparation itself was free from other ninyhydrin-reactive substances as shown on thin-layer electrophoresis-chromatography plates. N\(^2\)-(phosphonacetyl)-L-ornithine (PALO) was chemically synthesized as described elsewhere (3). The concentrations of PALO and phaseolotoxin were quantitatively estimated by assaying for phosphate as described previously (1). Phaseolotoxin concentration was also determined by the microbiological assay described previously (14). The two assays gave identical values. Standard stocks of phaseolotoxin (7 mM) and PALO (10 mM) were used throughout.

For OCTase assays the bacteria were grown in a minimal medium (14) with 0.5% succinate as a carbon source, at either 18 or 30°C, conditions under which phaseolotoxin is or is not produced, respectively. Cells were harvested in early stationary phase, washed three times with 50 mM Tris buffer, pH 8.0, and sonicated for 3 min in the cold with 1-s intermittent pulses. Cell pellets were removed by centrifugation at 12,000 \(\times g\) for 10 min at 4°C, and the supernatants were kept frozen (−20°C) until assayed. OCTase activity in the presence and absence of phaseolotoxin or PALO was measured by the conversion of [\(^{14}\)C]carbamoyl phosphate (dilithium salt; specific activity, 13.3 mCi/mmol; New England Nuclear Corp.) to [\(^{14}\)C]citrulline in 5 min as described by Hoogenraad (3). The amount of extract was adjusted by dilution to give 5 to 20% conversion of [\(^{14}\)C]carbamoyl phosphate. The reaction mixture (250 \(\mu\)l) contained 0.4 mM carbamoyl phosphate, 5 mM ornithine (Sigma Chemical Co.), enzyme extract, and various concentrations of phaseolotoxin or PALO in 50 mM Tris buffer, pH 8.0. The reaction was started by adding the enzyme extract to the above mixture. All assays were done in duplicate, and all experiments were repeated at least twice with new enzyme extracts. The crude sonic extracts showed no detectable activity above background level in the absence of added ornithine, indicating that all [\(^{14}\)C]carbamoyl phosphate conversion measured was due to citrulline and not to aspartate via the aspartate transcarbamoylase reaction utilizing endogenous aspartate present in the cell lysate.

The OCTase activities from *E. coli* and from

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**Fig. 1. OCTase activities in sonic extracts of toxigenic (NPPH3007 and HB36) and nontoxigenic (HB20) strains of *P. syringae* pv. *phaseolicola* and in *E. coli* K-12 strain N100 assayed in the presence of phaseolotoxin (A and B) and PALO (C). Enzyme assays were performed as described in the text. Results are expressed as a percentage of the OCTase activity in each extract measured in the absence of phaseolotoxin or PALO. Each point represents the average of duplicate (A and C) or triplicate (B) assays run in parallel (variation among replicate assays was 5 to 10%). The total \(^{14}\)C activity per assay tube was 135,000 cpm; the background activity in the absence of enzyme extract was 4,500 cpm. Radioactivity was measured in a liquid scintillation spectrometer with an external standard. (A) Symbols: ●, NPPH3007 grown at 18°C; ▲, HB36 grown at 18°C; △, N100 grown at 36°C; ■, HB20 grown at 18°C. Specific activities of OCTase in controls (no inhibitor added) were 0.04, 0.05, 0.06, and 0.03 \(\mu\)mol/min per mg of protein, respectively. (B) Symbols: ●, NPPH3007 grown at 18°C; ▲, HB36 grown at 18°C; ○, NPPH3007 grown at 30°C; △, HB36 grown at 30°C. Specific activities of OCTase in controls were 0.04, 0.05, 0.03, and 0.04 \(\mu\)mol/min per mg of protein, respectively. (C) Symbols: ●, NPPH3007 grown at 18°C; ▲, HB36 grown at 18°C; ○, NPPH3007 grown at 30°C; △, HB36 grown at 30°C. Specific activities of OCTase in controls were 0.05, 0.06, 0.05, and 0.07 \(\mu\)mol/min per mg of protein, respectively. Protein was assayed as described previously (2).**

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toxigenic *P. syringae* pv. *phaseolicola* strains NPPH3007 and HB36, cultured at 18°C, were compared over a range of phaseolotoxin concentrations from $7 \times 10^{-8}$ to $7 \times 10^{-6}$ M (Fig. 1A). Whereas the *E. coli* OCTase activity was strongly inhibited at low concentrations (50% inhibition at $2.7 \times 10^{-4}$ M), that from the *P. syringae* pv. *phaseolicola* strains was essentially insensitive to phaseolotoxin even at the highest concentrations tested. Furthermore, inhibition of the activity in the *E. coli* extract increased considerably after preincubation in the presence of phaseolotoxin, whereas this preincubation had no effect on the activity present in the extracts from toxigenic strains of *P. syringae* pv. *phaseolicola* grown at 18°C (Table 1). Preincubation has been reported to increase the inhibition of the OCTase from beans by phaseolotoxin, a mixture of compounds obtained from bean plants infected with *P. syringae* pv. *phaseolicola*, the exact chemical nature of which is presently unknown (4, 7, 11). Three other phaseolotoxin-producing *P. syringae* pv. *phaseolicola* strains (HB16, HB31, and HB33) were also surveyed for phaseolotoxin-insensitive OCTase activity after growth at 18°C. The inhibitions observed at a concentration of $7 \times 10^{-6}$ M phaseolotoxin were 18.11, and 18%, respectively. Thus, the occurrence of this unusual activity appears to be widespread among phaseolotoxin-producing strains of *P. syringae* pv. *phaseolicola*.

Natural isolates of *P. syringae* pv. *phaseolicola* which are unable to cause chlorotic symptoms in bean leaves (nontoxigenic) are frequently reported on in the literature. Such strains produce little or no phaseolotoxin (6, 14). In view of the above findings, it was of some interest to determine whether cell-free extracts from such strains also contained phaseolotoxin-insensitive OCTase activity. The OCTase activity from the phaseolotoxin-nonproducing strain HB20, cultured at 18°C, clearly differed from those detected in toxigenic strains in its sensitivity to phaseolotoxin (50% inhibition at $2 \times 10^{-4}$ M [Fig. 1A]). The enzyme activity from two other phaseolotoxin-nonproducing strains (HB-2 and HB-13) was also inhibited by phaseolotoxin (70 and 80%, respectively, at $7 \times 10^{-6}$ M).

Phaseolotoxin production by *P. syringae* pv. *phaseolicola* is temperature dependent, with 18°C being the optimal temperature and with little or no detectable toxin being produced at 30°C (6, 14). Although the biochemical basis of this regulation is not known, we considered the possibility that the presence of the toxin-insensitive OCTase activity in toxigenic strains may be linked to the production of phaseolotoxin. Figure 1B shows the results of two of several experiments in which the OCTase activities in sonic extracts of strains NPPH3007 and HB36 cultured at 18 and 30°C were assayed over a range of phaseolotoxin concentrations. In both strains the enzyme activity produced at 30°C differed dramatically from that produced at 18°C. The former was strongly inhibited by phaseolotoxin, whereas the latter was insensitive at the levels tested (Fig. 1B). Furthermore, the inhibition of the enzyme activity from phaseolotoxin-producing strains grown at 30°C increased after preincubation with the toxin (Table 1).

Since PALO is a known inhibitor of OCTase (3, 9), we examined whether the phaseolotoxin-insensitive and -sensitive OCTase activities detected in two phaseolotoxin-producing strains at 30 and 18°C were differentially inhibited by this compound. There was a marked difference in the sensitivity of the phaseolotoxin-resistant activity from NPPH3007 and HB36 grown at 18°C and the phaseolotoxin-sensitive activity from the same strains grown at 30°C (Fig. 1C). Whereas the concentrations of PALO needed for 50% inhibition of the sensitive enzyme activity from NPPH3007 and HB36 were $7 \times 10^{-7}$ and $1.3 \times 10^{-7}$ M, respectively, that for the insensitive enzyme activity from the same strains was $4 \times 10^{-6}$ M. Unlike phaseolotoxin, PALO inhibited the enzymes maximally without preincubation.

Our results show that phaseolotoxin-producing strains of *P. syringae* pv. *phaseolicola* grown at 18°C (optimal for phaseolotoxin production) contain an OCTase activity that is insensitive to phaseolotoxin. By contrast, the same strains grown at 30°C (temperature at which little or no phaseolotoxin is produced) contain an OCTase activity.
activity which is sensitive to the toxin. On the other hand, phaseolotoxin-nonproducing strains grown at 18°C contain phaseolotoxin-sensitive OCTase activity. The two OCTase activities found in phaseolotoxin-producing strains also differ substantially in their sensitivity to PALO. The occurrence and properties of the insensitive enzyme activity suggest that it plays a key role in the mechanism by which the phaseolotoxin-producing strains are immune to their own toxin. Our results, therefore, confirm and extend recent findings (A. R. Ferguson, J. S. Johnson, and R. E. Mitchell, FEMS Lett., in press) concerning the presence of phaseolotoxin-insensitive and -sensitive OCTase in phaseolotoxin-producing and -nonproducing strains of P. syringae pv. phaseolicola, respectively.

It is not presently known whether the two OCTase activities detected in the toxigenic strains after growth at 18°C reflect the presence of more than one structural gene for the enzyme or merely the post-transcriptional modification of a basic subunit under conditions permissive for toxigenesis. The possibility that either of these forms is a catabolic enzyme (13) is unlikely since P. syringae pv. phaseolicola, a member of the arginine dihydrolase-negative group of fluorescent pseudomonads (12), does not utilize arginine or citrulline for growth.

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